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Isotope Ratios of Cellulose from Plants Having Different Photosynthetic Pathways

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ABSTRACT

Hydrogen and carbon isotope ratios of cellulose nitrate and oxygen isotope ratios of cellulose from C_3, C_4, and Crassulacean acid metabolism (CAM) plants were determined for plants growing within a small area in Val Verde County, Texas. Plants having CAM had distinctly higher deuterium/hydrogen (D/H) ratios than plants having C_3 and C_4 metabolism. When hydrogen isotope ratios are plotted against carbon isotope ratios, each photosynthetic mode separates into a distinct cluster of points. C_4 plants had many D/H ratios similar to those of C_3 plants, so that hydrogen isotope ratios cannot be used to distinguish between these two photosynthetic modes. *Portulaca mundula*, which may have a modified photosynthetic mode between C_4 and CAM, had a hydrogen isotope ratio between those of the C_4 and CAM plants. When oxygen isotope ratios are plotted against carbon isotope ratios, no distinct clustering of the C_4 and CAM plants occurs. Thus, oxygen isotope ratios are not useful in distinguishing between these metabolic modes. A plot of hydrogen isotope ratios *versus* oxygen isotope ratios for this sample set shows considerable overlap between oxygen isotope ratios of the different photosynthetic modes without a concomitant overlap in the hydrogen isotope ratios of CAM and the other two photosynthetic modes. This observation is consistent with the hypothesis that higher D/H ratios in CAM plants relative to C_3 and C_4 plants are due to isotopic fractionations occurring during biochemical reactions.

A large body of research has demonstrated that stable carbon isotope ratios of plants are related to photosynthetic pathways (1, 2, 19). C_3 plants, which fix CO_2 via ribulose bisphosphate carboxylase (1), have $\delta^{13}C$ values (see "Materials and Methods" section for definition of δ) that average about −25‰, while C_4 and CAM plants, which fix CO_2 via P-enolpyruvate carboxylase (1), have average $\delta^{13}C$ values of about −13‰ (1). Thus, carbon isotope ratios can be used to distinguish C_3 plants from CAM and C_4 plants. Differences of $\delta^{13}C$ values among species within a given photosynthetic group are caused at least in part by gas exchange efficiency and factors associated with the leaf surface (10).

It has been demonstrated that hydrogen isotope ratios can be used to distinguish CAM plants from C_3 and C_4 plants (20, 21, 25). In one study, cellulose nitrate prepared from CAM plants collected in Riverside County, California, had average δD values of +56 ± 30‰ (n = 11) while cellulose nitrate of C_3 and C_4 plants growing at the same site had average δD values of −69 ± 35 (n = 9) and −27 ± 4‰ (n = 2), respectively (20). In a study of greenhouse-grown plants, the average δD value of cellulose nitrate of CAM plants was +30 ± 19‰ (n = 20), while C_3 and C_4 plants had average δD values of −80 ± 35 (n = 10) and −25 ± 2‰ (n = 2), respectively (21). In both cases, δD values of CAM plants did not overlap with those of C_3 and C_4 plants. Oxygen isotope ratios, however, could not be used to distinguish CAM from C_3 and C_4 plants in either of these studies.

Previous reports on differences of hydrogen isotope ratios between C_3 and C_4 plants are conflicting (18, 25). Ziegler *et al.* (25) reported large differences in hydrogen isotope ratios between C_3 and C_4 plants, with C_4 plants being enriched in deuterium relative to C_3 plants. Smith and Epstein (18), however, observed no differences between hydrogen isotope ratios of C_3 and C_4 plants. One of the problems with both of these earlier reports was that isotope analysis was done on total plant matter. Different chemical components of plant matter can have different isotope ratios (16). Thus, differences in chemical composition between plants might contribute to differences in δD values and $\delta^{18}O$ values. Furthermore, a large proportion of hydrogen in organic matter is exchangeable with water (9), and thus, some routine treatments of samples, such as rinsing with water, can alter their hydrogen isotope ratios. Isotopic analysis of plant cellulose and cellulose nitrate eliminates these variables because measurements are made on only one chemical component and exchangeable hydroxyl hydrogens are eliminated by nitration (9).

Another complicating factor in the study of oxygen and hydrogen isotopes in plants is that oxygen and hydrogen isotope ratios of plants are not influenced only by plant physiological behavior but also by climate and the $^{18}O/^{16}O$ and D/H ratios of meteoric water. Variations in the isotopic composition of meteoric water ranging from about −40 to +6‰ for $\delta^{18}O$ values and from about −320‰ to +50‰ for δD values have been reported (5). In general, meteoric water from warmer and drier climates has higher $\delta^{18}O$ and δD values than meteoric water from cooler and more humid regions (12). Plant oxygen and hydrogen isotope ratios are also influenced by the effects of climate on transpiration. Leaf water from plants growing in drier climates has higher δD and $\delta^{18}O$ values relative to local meteoric water than leaf water from plants growing in more humid climates (11, 23). To eliminate the effect of variability of isotope ratios of meteoric water and the effect of different climates on the plant oxygen and hydrogen isotope ratios, plants must be sampled within a restricted area, thus making it more likely that meteoric water and climate are the same for all plants being compared. Only then can differences in D/H and $^{18}O/^{16}O$ ratios among groups of plants representing the different photosynthetic modes be ascribed to distinctive physiological and biochemical processes.

Recent analysis of cellulose nitrate and cellulose from C_3 and C_4 plants growing at one site in Riverside County, California and in a greenhouse showed that, in each case, hydrogen and oxygen isotope ratios of C_4 plants were slightly higher than that of C_3 plants (20, 21). However, in these reports only two C_4 species were represented in each of the two sample sets, and thus, no firm conclusions could be drawn. In this study we report results of isotopic analysis of cellulose nitrate and cellulose from a sample set of 35 species composed of 13 C_4, 12 C_3, and 10 CAM species, all growing in close proximity in a well defined small drainage system.

MATERIALS AND METHODS

Plants were collected near the Highway 90 bridge over the Pecos River in Val Verde County, Texas. The plants sampled were growing close to one another to maximize the probability that they had access to meteoric water with the same isotopic signature. Stem and leaf material were collected from three separate individual plants or plant populations (in the case of small plants) for each of the species selected. The triplicate samples provided opportunity for replicating isotope measurement of individual species. After the plants were identified, they were dried at 50°C for several days.

Dried plant material was ground to fine powder in a Wiley mill. Cellulose was extracted by the method of Wise (24). Oxygen isotope ratios of cellulose were determined by the method of Rittenberg and Ponticorvo (17) as modified by Burk (4). Carbon and hydrogen isotope ratios of cellulose nitrate prepared as described elsewhere (6) were determined by a modified version of the Stump and Frazer method (16, 22). Isotope ratios are expressed as δ values:

$$\delta(‰) = \left[\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right] \times 1000$$

where R represents $^{18}O/^{16}O$ for oxygen, D/H for hydrogen, and $^{13}C/^{12}C$ for carbon. The standards were SMOW[1] for oxygen and hydrogen and the PDB for carbon. The precisions of the isotopic analyses were ±2‰ for δD, ±0.5‰ for $\delta^{18}O$ and ±0.2‰ for $\delta^{13}C$. A limited number of replicate measurements were made for selected species from each of the photosynthetic types.

RESULTS

Carbon isotope ratios of plants sampled in this study are similar to those expected based on previously reported measurements for plants representing different photosynthetic modes (1, 2, 19), with C_3 plants having $\delta^{13}C$ values ranging from −22.2 to −26.1‰ and CAM and C_4 plants having $\delta^{13}C$ values between −10.6 to −13.8‰ (Table I). Thus, all CAM plants sampled in this study were operating in the CAM mode. The relative distribution of hydrogen isotope ratios among the plants representing the different photosynthetic types is also similar to previous reports (20, 21). Values of δD ranged from −71 to −9‰ for C_3 plants, −53 to −20‰ for C_4 plants, and +34 to +66‰ for CAM (Table I). δD values for CAMs (51 ± 10‰, $n = 12$) are discrete from those of C_3 and C_4 plants, while those for C_3 (−42 ± 16‰, $n = 16$) and C_4 (−33 ± 12‰, $n = 16$) plants show considerable overlap (Table II and Fig. 1). Thus, it is unlikely that hydrogen isotope ratios will be useful to distinguish between these two photosynthetic pathways. The oxygen isotope ratios of the plants sampled showed more distinct patterns with less variability than previously reported for plants representing the different photosynthetic pathways (20, 21). Values of $\delta^{18}O$ ranged from +23.5 to +29.2‰ for C_3 plants, +32.5 to +37.0‰ for C_4 plants, and +22.6 to +35.4‰ for CAMs (Table I). The average values were highest for C_4 plants (+34.7 ± 1.4‰, $n = 16$), followed by CAM (+29.7 ± 3.1‰, $n = 12$) and C_3 plants (+26.8 ± 1.9‰, $n = 16$) (Table II). The $\delta^{18}O$ values of the C_4 plants were distinctly separate from those of the C_3 plants, whereas the $\delta^{18}O$ values of C_4 plants and CAM plants showed some overlap (Fig. 2).

[1] Abbreviations: SMOW, standard mean ocean water; PDB, belemnite from the Peedee formation of South Carolina.

Table I. *Species, Photosynthetic Mode, Carbon and Hydrogen Isotope Ratios of Cellulose Nitrate and Oxygen Isotope Ratios of Cellulose for each Species Sampled*

In some cases, more than one specimen was analyzed. CAM plants were identified by their succulence, C_4 plants by Kranz anatomy, and C_3 plants by their lack of succulence and Kranz anatomy.

Species	Mode	$\delta^{13}C$	δD	$\delta^{18}O$
Acacia belandieri	C_3	−25.0	−49	+23.5
Acacia rigidula	C_3	−24.2	−56	+23.5
Cassia lindheimeriana	C_3	−25.7	−45	+28.3
Fouquieria splendens	C_3	−26.1	−47	+27.0
Fraxinus greggii	C_3	−23.3	−23	+26.6
		−22.9	−17	+25.0
		−23.9	− 9	+26.2
Jatropa dioica	C_3	−24.2	−52	+24.9
Lippia graveolens	C_3	−26.1	−71	+28.8
Penstemon bacharifolius	C_3	−24.0	−42	+27.6
		−25.6	−65	+28.2
		−25.8	−50	+29.2
Prosopis glandulosa	C_3	−23.6	−44	+24.5
Selagenella lepidophylla	C_3	−25.9	−31	+28.9
Selagenella underwooddii	C_3	−25.7	−40	+27.8
Dasylirion texanum	C_3	−22.2	−28	+28.3
Aristida wrightii	C_4	−12.6	−53	+32.5
		−11.8	−45	+32.6
Bothriochloa sacchariodes	C_4	−11.4	−36	+35.1
Bouteloua curtipendula	C_4	−12.1	−34	+32.5
Bouteloua hirsuta	C_4	−12.4	−42	+37.0
		−12.5	−29	+35.2
Digitaria californica	C_4	−12.1	−38	+34.5
Erioneuron pilosum	C_4	−12.7	−25	+33.9
Heteropogon contortus	C_4	−11.6	−43	+33.8
Leptoloma cognatum	C_4	−11.0	−21	+36.7
Panicum hallii	C_4	−12.1	−40	+35.8
Pappophorum bicolor	C_4	−12.1	−42	+34.9
Portulaca mundula	C_4	−11.9	− 1	+33.9
Sporobolus cryptandrus	C_4	−12.4	−33	+34.8
Setaria viridis	C_4	−11.4	−20	+36.7
		−11.5	−34	+34.4
Agave lecheguilla	CAM	−11.0	+51	+22.6
Echinocereus enneacanthus	CAM	−10.9	+60	+31.1
		−12.5	+44	+35.4
Echinocereus triglochidiatus	CAM	−10.6	+34	+29.2
Ferocactus hamataeanthus	CAM	−12.8	+52	+29.7
Opuntia edwardsii	CAM	−13.8	+36	+28.7
Opuntia leptocaulis	CAM	−12.2	+57	+32.1
Opuntia lindheimeri	CAM	−11.5	+54	+27.9
Opuntia phaeocantha	CAM	−12.3	+39	+27.9
Yucca torreyi	CAM	−11.6	+63	+30.5
		−12.0	+66	+31.8
Yucca baccata	CAM	−13.5	+57	+29.0

Table II. *Average δ Values of each Photosynthetic Mode Sampled in this Study*

Photosynthetic Mode	Average Isotope Ratios		
	$\delta^{13}C$	δD	$\delta^{18}O$
C_3	−24.6 ± 1.3‰	−42 ± 16‰	+26.8 ± 1.9‰
C_4	−12.0 ± 0.5‰	−33 ± 12‰	+34.6 ± 1.4‰
CAM	−12.1 ± 1.0‰	+51 ± 10‰	+29.7 ± 3.1‰

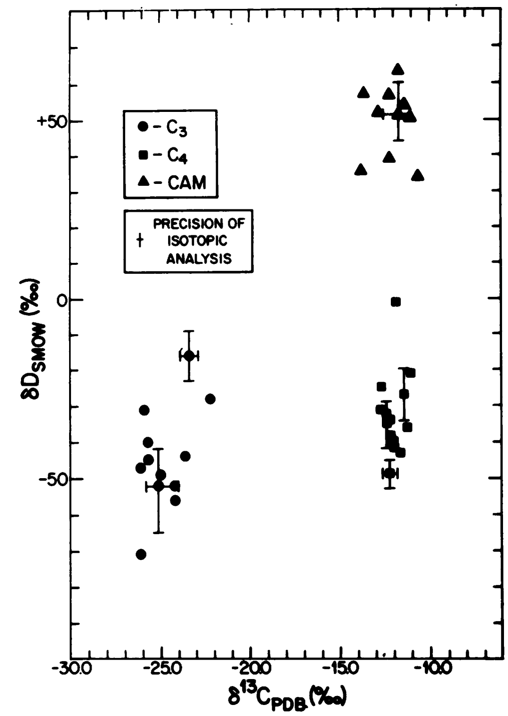

FIG. 1. Relationship between δD and $\delta^{13}C$ values of C_3, C_4, and CAM plants. Points with bars indicate the mean and range of multiple measurements for different specimens of selected species.

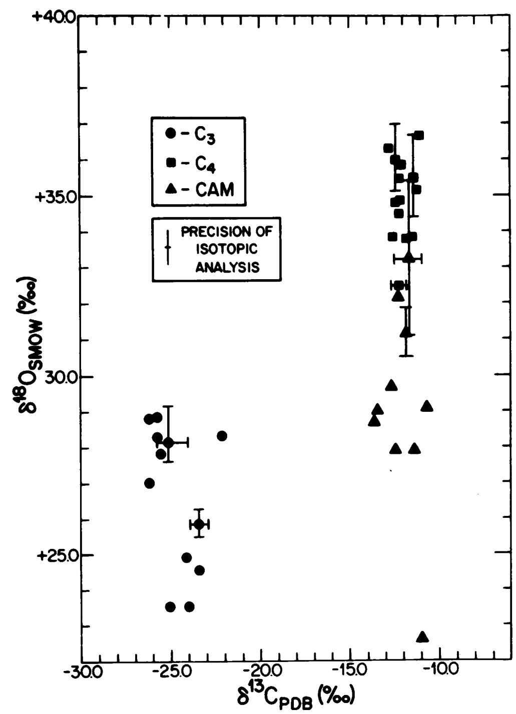

FIG. 2. Relationship between $\delta^{18}O$ and $\delta^{13}C$ for the three photosynthetic modes. Points with bars indicate the mean and range of multiple measurements for different specimens of selected species.

DISCUSSION

Clustering of each photosynthetic type relative to their δD and $\delta^{13}C$ values (Fig. 1) is similar to but more pronounced than that reported previously (20, 21), with CAM plants in the upper right quadrant, C_4 in the lower right quadrant, and C_3 plants in the lower left quadrant. The C_4 group has one outlying point with an exceptionally high δD value of −1‰ (Fig. 2). This value was obtained for cellulose nitrate of *Portulaca mundula* (Table I), which is a succulent C_4 plant. Other species of this genus, namely *P. olearaceae* and *P. grandiflora*, have characteristics of CAM plants, such as acid flux and succulence, as well as characteristics of C_4 plants, such as Kranz anatomy (13, 14). Because *P. mundula* is also a succulent C_4 species with Kranz anatomy, it quite possibly has the same modified photosynthetic mode as *P. oleraceae* and *P. grandiflora*. This may have caused its δD value to be intermediate between those of the C_4 and CAM groups. If this is the case, hydrogen isotope ratios may be sensitive to shifts from C_4 to CAM. This type of shift would not affect the carbon isotope ratio, since the primary carboxylation reaction is mediated by P-enolpyruvate carboxylase for both photosynthetic pathways.

Oxygen isotope ratios of cellulose relative to the $\delta^{13}C$ values of cellulose nitrate (Fig. 2) do not separate the photosynthetic types into separate clusters as completely as did the hydrogen and carbon isotope ratios (Fig. 1). Thus, oxygen isotope analysis cannot be used to distinguish CAM plants from C_4 plants. One exceptional point (lower right quadrant) in Figure 2 deserves further discussion. It represents *Agave lechequila*, a CAM species that had an unusually low $\delta^{18}O$ value relative to the other CAM plants. We had observed previously that another *Agave* species, *A. deserti*, had low $\delta^{18}O$ values relative to other CAM species growing in the same area (20). Although these two *Agave* species are leaf succulents, their low $\delta^{18}O$ values probably are not due solely to their physiognomy, as suggested earlier (20), since two other leaf succulent species analyzed in the present study, *Yucca bacatta* and *Y. torreyi* (Table I) did not have low $\delta^{18}O$ values. Some other hypothesis must be advanced to explain the low $\delta^{18}O$ values of these *Agave* species.

Figure 3 shows the relationship between the δD values of cellulose nitrate and the $\delta^{18}O$ values of cellulose extracted from plants sampled in this study. The data points are separated into

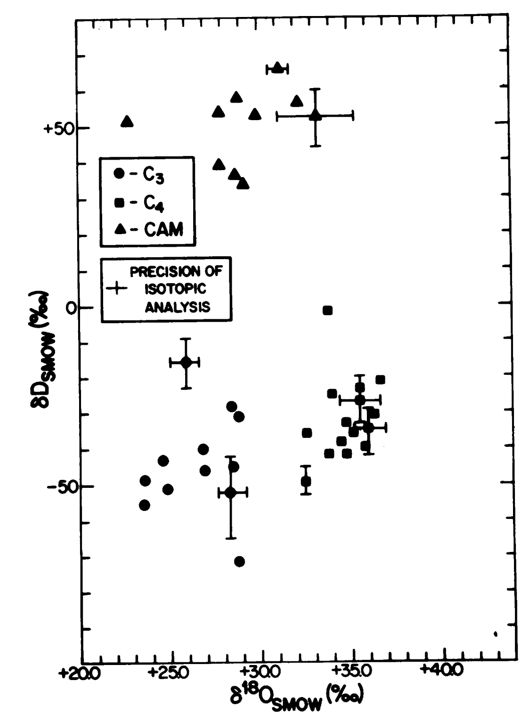

FIG. 3. Relationship between δD and $\delta^{18}O$ for the three photosynthetic modes. Points with bars indicate the mean and range of multiple measurements for different specimens of selected species.

three clusters representing the C_3, C_4, and CAM photosynthetic types, as was observed in the δD *versus* δ^{13}C plot (Fig. 1). Three factors influence the relationship between the δD value of cellulose nitrate and the δ^{18}O value of cellulose in a plant. The first is the relationship between the δD and δ^{18}O values of the groundwater absorbed by the plant through its roots, which is given by the equation $\delta D = 8 \times \delta^{18}O + 10‰$ (the meteoric water line equation) (5). The second factor relates to the isotopic fractionation that occurs during evapotranspiration, in which the leaf water becomes enriched in deuterium and in ^{18}O relative to groundwater (15). The relationship between the δD and δ^{18}O values of leaf water from plants undergoing evapotranspiration is approximately linear with a slope of about 3 (15). The third factor which influences the relationship between the δD value of cellulose nitrate and δ^{18}O value of cellulose are the biochemical isotopic fractionations associated with carbohydrate and cellulose synthesis (8). If the elevated hydrogen isotope ratios in the CAM group are due to evapotranspiration, then the oxygen isotope ratios of cellulose from CAM plants should also be distinctly elevated. This is because evapotranspiration, which causes enrichment of deuterium, also enriches plant water in ^{18}O (11), with the ^{18}O-enrichment subsequently being passed on to cellulose (7). Joint enrichment of D and ^{18}O in CAM cellulose nitrate and cellulose was not observed in this study. The δD values of the CAM group are elevated relative to the rest of the samples measured without a concomitant increase in δ^{18}O values of cellulose (Fig. 3). Therefore we conclude, as we did previously (20), that δD values in CAM plants are elevated due to biochemical reactions.

The C_4 group shows elevated δD and δ^{18}O values relative to the C_3 plants. One explanation might be that C_4 plants produce their cellulose at a different time of the year and thus use meteoric water with a different isotopic composition than that used by C_3 plants. This explanation seems unlikely because an increase in the δ^{18}O values of meteoric water will be accompanied by an increase in the δD values by a factor of 8 (5). In our sample set, however, the hydrogen isotope ratios of cellulose nitrate of C_4 plants are only slightly higher than those of the C_3 group, while the δ^{18}O values of cellulose from the two groups differ by large amounts. Furthermore, all plants sampled showed evidence of being essentially opportunistic in exhibiting new growth in response to late summer rains just prior to collection. A second explanation is that differences in δD and δ^{18}O values between C_3 and C_4 plants are related to evapotranspiration. A regression analysis between the δD values of cellulose nitrate and δ^{18}O values of cellulose from C_3 and C_4 plants gives a line with a slope of 1.4 (intercept = -80, $r = 0.43$) which approaches the slope of the δD *versus* δ^{18}O line of leaf water from plants which are undergoing evapotranspiration (15). Regression characteristics from previously published results (20) were similar (slope = 1.5, intercept = -94.5, $r = 0.50$). Thus, it seems likely that differences in oxygen and hydrogen isotopes in C_4 plants relative to C_3 plants are related to evapotranspiration and not biochemical reactions or differences in the water available for plant growth. Recent comparative field studies demonstrated that C_4 plants are less sensitive to a decrease in RH when compared to C_3 plants (3). C_4 plants did not show a decrease in photosynthesis with a reduction in RH whereas C_3 plants did. Thus, C_4 plants may have a considerable amount of cellulose synthesized under drier conditions when plant water would be more enriched in deuterium and oxygen relative to meteoric water; conversely, C_3 plants may not synthesize much cellulose unless conditions are more humid.

The differences in δ^{18}O and δD values observed among species and/or individual plants within a given photosynthetic type may reflect processes associated with gaseous exchange, water use efficiency, and tolerance to moisture stress that are more subtle than those that separate the major photosynthetic pathways (10, 11).

CONCLUSIONS

Our measurements agree with previously published results in that a clear discrimination existed among C_3, C_4, and CAM photosynthetic pathways when hydrogen and carbon isotope ratios of cellulose nitrate were considered. These measurements might also be sensitive to plants which have modified photosynthetic modes between C_4 and CAM, as may be the case for *P. mundula*. A large overlap in oxygen isotope ratios existed between CAM and the other two photosynthetic pathways without an overlap in hydrogen isotope ratios of CAM plants relative to C_3 and C_4 plants. This indicates that deuterium enrichment in CAM plants as compared to C_3 and C_4 plants is due to isotopic fractionations occurring during biochemical reactions and not during evapotranspiration, as has been suggested (25). Cellulose from C_4 plants had distinctly different oxygen isotope ratios compared to cellulose from C_3 plants. Our data are consistent with the hypothesis that as conditions become drier, C_4 plants continue cellulose synthesis while cellulose synthesis for C_3 plants is inhibited. Thus, cellulose in C_4 plants would be synthesized under drier conditions when plant water would be enriched in deuterium and ^{18}O. This hypothesis can be tested by growing C_4 plants and C_3 plants under controlled arid and humid conditions and then examining the relationships between their δD and δ^{18}O values.

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